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PRINCIPAL INVESTIGATOR: Benjamin B. Mull

CONTRACTING ORGANIZATION: UT MD Anderson Cancer Center
Houston, TX 77030

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14. ABSTRACT: The adverse effects of cancer chemotherapy are widely recognized. Hair loss, gastrointestinal discomfort, lethargy and anorexia are quite common. The cause for these events is the nonspecific nature of current cancer treatment agents. Cytotoxic drugs, while effective at killing proliferating tumor cells, also target normal dividing cells. It is the purpose of this study to develop a proven in vitro strategy to protect normal dividing tissues using a cytostatic agent, UCN-01. There reversible arrest of normally dividing tissues in mice will be examined for improved tolerance of chemotherapeutics. This protective effect will also be evaluated in mice bearing orthotopically implanted breast tumors.					
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Introduction:

The side effects of cancer chemotherapy are well known. The purpose of this study is to determine if a protective protocol developed in cultured cells will be effective in an animal model system. UCN-01, a cytostatic drug and kinase inhibitor currently in preclinical trials, has been demonstrated in our laboratory to arrest normal cells but not tumor cells in a reversible fashion. The temporarily arrested normal cells are able to evade many of the toxicities of chemotherapeutic agents. The proposal reported upon here is to determine if UCN-01 can be used in a mouse model system to protect the normal dividing cells of the body from these toxic effects by placing them in a reversible state of arrest. The work in the first year of this project demonstrated that the dividing cells of the intestinal epithelium can be reversibly arrested by UCN-01. The work in the second award period focused on minimizing the antagonistic effect of the drug carrier (DMSO) on the action of UCN-01. Technical difficulties encountered during the tissue fixation procedure were also addressed and resolved. During this phase of the work, UCN-01 was evaluated as a protective agent against the toxic effects of 5-fluorouracil. It was demonstrated that a 48 hour pretreatment with UCN-01 resulted in a significant increase in platelets following a course of 5-FU. Other time points will be evaluated for short term apoptotic effects. The final aspect, treating tumor bearing mice with this protocol, will be carried out during a one-year no-cost extension period, which has already been applied for.

Body:

The work reported to date for this project have satisfied the first two parts of the Statement of Work. We have successfully created a model system to follow to cell cycle kinetics of the gut epithelium of nude (athymic) mice using bromodeoxyuridine (BrdUrd) labeling and flow analysis. Using this system, we have demonstrated a cell cycle arrest in the small bowel following injections of 0.63 mg/kg to 10 mg/kg UCN-01. We have also shown that this arrest is reversible; the epithelial cells of the small bowel return to their normal cycling profile 2 weeks following UCN-01 administration, and by 4 weeks are in a hyperproliferative state, presumably to repopulate the gut after the arrest. This past year's work has focused on the last two parts of the SOW. Aim 3 was to demonstrate an appreciable difference in tolerance of chemotherapeutics in mice "protected" by temporary UCN-01 arrest. We chose to use 5-fluorouracil as the cytotoxic agent for these experiments, as it is known to be highly toxic in the gut. The initial experiment comprised of 6 cohorts of 5 mice each (30 total). 15 mice were given 5 mg/kg UCN-01 via intramuscular injection; the other 15 were given DMSO carrier at the appropriate volume/weight. The mice were given 2 days to allow the UCN-01 to take effect. The cohorts were then injected as shown in the table below.

Cohort	UCN-01/DMSO	5-FU/PBS
1	UCN-01	PBS X 5
2	UCN-01	25 mg/kg 5-FU X 5
3	UCN-01	35 mg/kg 5-FU X 5
4	DMSO	25 mg/kg 5-FU X 5
5	DMSO	35 mg/kg 5-FU X 5
6	DMSO	PBS X 5

The mice were injected with either 5-FU or PBS daily for five days in a row. Weights were followed each day, and are shown in figure 1. Unfortunately, no difference was observed between either UCN-01/5-FU cohort and its matching "unprotected" group with 5-FU alone. It was also unexpected that no mortality was seen in the high dose 5-FU groups; these doses were based on previous experiments in which 80% of the mice (C3H strain) at 35 mg/kg/day X 5 had died.

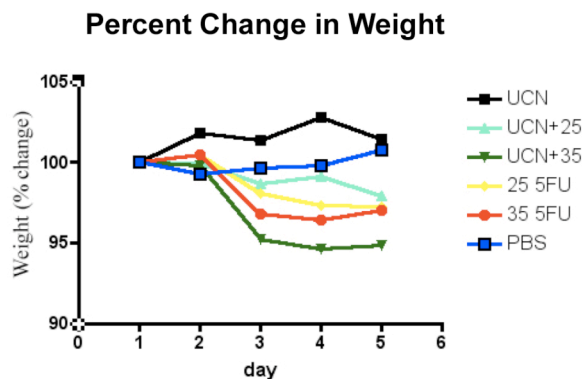


Figure 1. Percent change in daily weight following treatment with 5-fluorouracil in mice with and without UCN-01 protection

The lack of any mortality in our experiment was curious. We decided to do a second experiment at only the high dose of 5-FU to better understand the effects of our treatment. For this experiment, we would not only follow the weights of the animals but also collect blood at sacrifice to see how our treatment is affecting the hematopoietic system as well as the liver. As before, 15 mice were injected with 5mg/mg UCN-01 and another 15 controls were given appropriate volumes of DMSO

based on weight. Two days later, the mice were treated with 5-FU or control PBS, but only at the high (35 mg/kg) dose; this was again done for 5 consecutive days. The breakdown of the groups is listed in the table below

Cohort	UCN-01/DMSO	5-FU/PBS
1	UCN-01	PBS X 5
2	UCN-01	35 mg/kg 5-FU X 5
3	UCN-01	35 mg/kg 5-FU X 5
4	DMSO	35 mg/kg 5-FU X 5
5	DMSO	35 mg/kg 5-FU X 5
6	DMSO	PBS X 5

As before, weights were followed every day (Figure 2). Two days after the final 5-FU/PBS injection, groups 1, 2, 5, and 6 were sacrificed. Gut tissue was harvested and formalin-fixed for histochemical analysis. Blood was collected in two portions; whole blood for complete blood counts (CBC) in EDTA tubes to prevent clotting, and serum for liver damage assessment. The blood counts for the four groups (UCN-01, UCN-01+PBS, 5-FU, PBS) as well as the for white blood cells, hemoglobin and platelets are shown in figure 3. While no significant differences in weight were observed in the “protected” 5-FU group and 5-FU alone, the protected group did have significantly higher platelets

Daily Percent Change in Mouse Weight

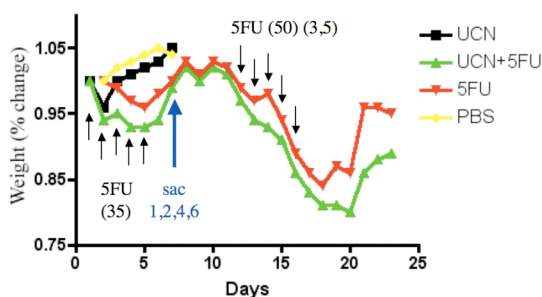


Figure 2. Daily percent change in mouse weights for the second experiment. 10 mice in each of 6 groups. Cohort 1, 2, 4, and 6 were sacrificed after one course of 5-FU treatment (35 mg/kg/day X 5). Groups 3 and 5 were given a subsequent treatment of 50 mg/kg/day X 5 and sacrificed

than the 5-FU alone group. Liver enzymes for the four groups are also shown in figure 3. No differences between groups 2 and 5 were observed. However, it is curious that AST and ALT are “elevated” in the PBS mice (group 6). We do not understand this result, as increased liver enzymes in the blood suggest hepatocyte damage. How the mice receiving only control injections could display the most damage is not yet understood. It is likely that 5-FU and/or UCN-01 in the serum may interfere with the detection of liver enzymes. If so, histological analysis of the liver will be

required. The formalin-fixed intestinal tissue was subjected to immunofluorescent staining for exposed DNA ends (TUNEL) to assess the degree of apoptosis due to our cytotoxic treatment. However, as shown in figure 4, no significant apoptosis was seen in any sample. Given the known toxicity of 5-FU and the weight loss experienced by the mice during treatment this result was unexpected. Also puzzling was the continued lack of mortality due to 5-FU. To further explore this, we took the remaining cohorts (3 and 4) and let them recover from treatment for 4 weeks. We then did a much higher dose regimen of 5-FU; 50mg/kg/day X 5 days for group 4, and mg/kg UCN-01 followed by 50mg/kg/day 5-FU X 5 days (started 48 hours after UCN-01 administration) for group 3. Weights were followed daily (seen as days 12-24 in figure 2). As before, no mortality was observed in either group. 2 days after the final 5-FU injection, some of the mice had lost over 25% of their body weight, so they were all sacrificed. The mice were severely dehydrated and no blood was collected; gut tissue was removed and formalin-fixed for

histochemical analysis. This data was presented to the clinicians in our group, and two suggestions were made. First, concerning the lack of apoptosis in our gut samples, it was suggested that peak cell death would occur within 24 hours of 5-FU administration. Therefore, while our protocol of daily injections and then 48 hours later harvesting tissue is appropriate to model changes in the blood-producing cells of the bone marrow, it would cause us to miss the peak of apoptosis. We are planning a future experiment using a single bolus dose of 5-FU and sacrifice 24 hours later to address this issue. As for the lack of mortality, it was suggested that the athymic nature of the nude mice is a concern. The lack of T-cells in the nude mice prevents them from mounting an appropriate inflammatory response to our cytotoxic agent. This could partly explain how the mice were able to tolerate extremely high doses of 5-FU and account for the lack of elevated liver enzymes in the blood. To better understand this aspect of the project, we are currently repeating the above experiments in an immune competent 129 mouse model system.

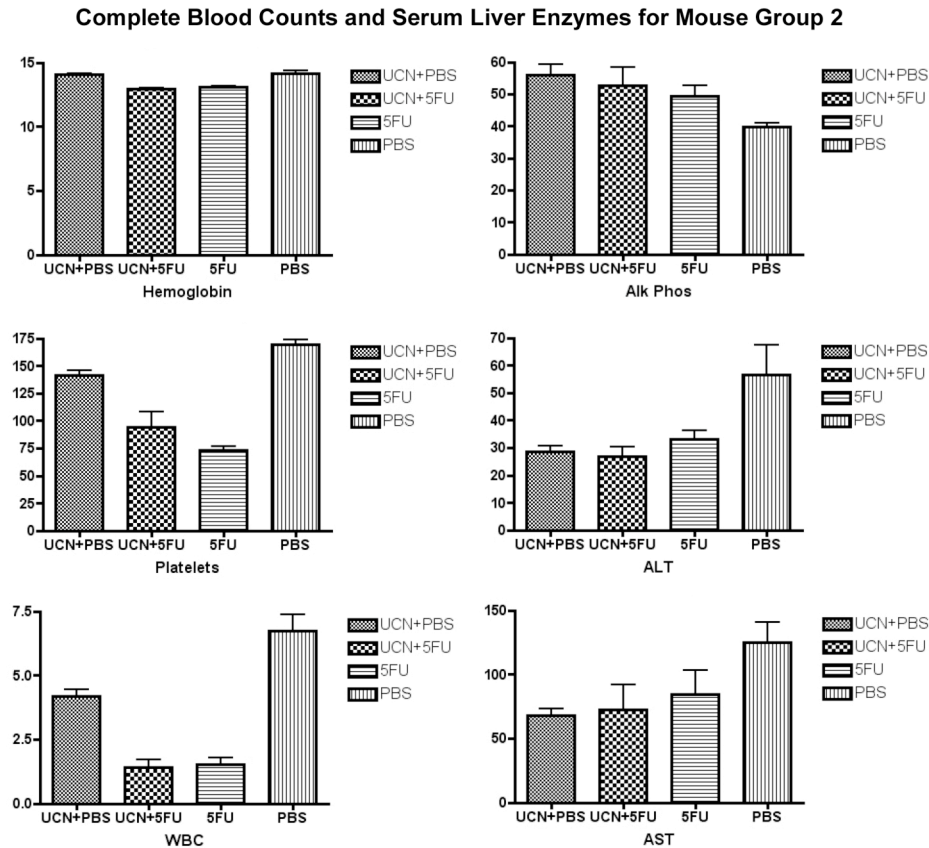


Figure 3. Hemoglobin, platelets and white blood cell counts from whole blood collected after 35 mg/kg/day 5-FU treatment. Platelet counts show a significant improvement with UCN-01 protective pretreatment. Serum liver enzymes indicate no apparent toxicity due to UCN-01 administration.

Due to a leave of absence last year and the technical problems encountered in the second year, we filed a one year no-cost extension of this grant to complete the work, using the altered timeline below:

- Task 1.* To demonstrate the arresting effects of UCN-01 in the mouse small bowel epithelium (COMPLETED).
- Task 2.* To demonstrate reversibility of UCN-01 cytostatic effects and determine the optimal time course for treatment (COMPLETED).
- Task 3.* To demonstrate improved tolerance of chemotherapeutics in mice Receiving the arresting agents (Months 28-40)
 - a. Compare adverse effects of cytotoxic drugs in protected vs. unprotected mice (COMPLETED).
 - b. Increase doses of cytotoxic drugs in protected mice until adverse effects are similar to unprotected mice. Create new MTD in protected mice (IN PROGRESS, Months 36-40)
- Task 4.* To treat tumor-bearing mice with and without the protection strategy (from task 3) to show improved course of treatment (Months 40-48).

Key Research Accomplishments:

- Demonstrated a protective effect of UCN-01 pretreatment against the hematopoietic toxicity of 5-FU, seen as an increase in platelets in whole blood.
- Refined the time course of UCN-01 arrest.
- Evaluated the hepatotoxicity of 5-FU with and without UCN-01 pretreatment via serum liver enzymes.

Reportable outcomes:

No reportable outcomes as of this writing.

Conclusions:

The previous award period provided the timeframe for UCN-01 arrest and release. In the current work period, we have used this protocol to assess the protective effect of UCN-01 pretreatment against the cytotoxic effects of chemotherapeutics, specifically 5-fluorouracil (5-FU). It was demonstrated that following a 48 hour pretreatment of UCN-01, the loss of platelets due to a high dose of 5-FU was diminished. TUNEL analysis of tissues from these mice showed no appreciable effect of UCN-01; a shorter time period will be evaluated to better understand the apoptotic consequences of UCN-01 protection. During the no-cost extension period, this protocol will be followed to evaluate the protective effect in mice bearing implanted breast tumors.